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Bruce D. Schanbacher
USDA-ARS

Hugh F. English
USDA-ARS

Douglas Gross
USDA-ARS

Richard J. Santen
USDA-ARS

Michael F. Walker
USDA-ARS

See next page for additional authors

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Animal Model of Isolated Gonadotropin Deficiency

I. Hormonal Responses to LHRH Immunoneutralization

BRUCE D. SCHANBACHER, HUGH F. ENGLISH, DOUGLAS GROSS, RICHARD J. SANTEN, MICHAEL F. WALKER, AND RICHARD E. FALVO

From the Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska, the Department of Medicine, The Milton S. Hershey Medical Center, Hershey, Pennsylvania, The School of Medicine, University of California, Davis, California and The School of Medicine, Southern Illinois University, Carbondale, Illinois

Five intact male mongrel dogs, characterized by an episodic secretory pattern of LH and normal serum testosterone concentrations, were actively immunized against LHRH by subcutaneous injections of 200 μg of a LHRH-human serum albumin conjugate at 0, 4, and 8 weeks. After 12 weeks, two dogs having the highest antibody titers to LHRH (25% and 51% binding of ^{125}I-LHRH in serum diluted 1:1000 B/Bo) had low to nondetectable serum concentrations of LH and testosterone, whereas serum FSH concentrations were significantly reduced in only one of these dogs. Immunocytochemical techniques showed that the pituitaries of these same two dogs had smaller and fewer LH immunoreactive gonadotropes than did the pituitaries of another three immunized-nonaffected dogs or of the five nonimmunized control dogs. The two LHRH-immunized dogs characterized by hypogonadotropism also had reduced testis (4.0 and 4.0 g) and prostate (2.1 and 1.7 g) weights when compared to control dogs (testis: 12.1 ± 1.0 g and prostate: 9.2 ± 1.9 g). LHRH antibody titers in three immunized dogs were demonstrable (8.1, 9.8, and 14.2% B/Bo), but effects on LH, FSH, and testosterone concentrations, pituitary gonadotropes, and reproductive tissue weights were not apparent. The similarity in hormonal and tissue responses observed between dogs effectively immunized against LHRH and men with isolated gonadotropin deficiency suggests that the LHRH-immunized dog may provide a suitable experimental model for the study of patients with isolated gonadotropin deficiency.

Key words: hypogonadotropic-hypogonadism, luteinizing hormone releasing hormone; pituitary, serum LH, serum FSH, serum testosterone, dog, immunohypophysectomy.

Isolated gonadotropin deficiency occurs as an autosomal dominant syndrome in man and produces sexual infantilism as a result of androgen deficiency (Santen and Paulsen, 1973a,b; Santen and Kulin, 1981). Patients with this disorder have been used to study the effects of gonadotropin replacement therapy on Leydig cell and spermatogenic function (Paulsen et al, 1970; Sherins et al, 1977). However, the constraints of clinical research limit the scope of such studies. An animal model of this syndrome would allow more intensive investigation with gonadotropin replacement therapy under a variety of experimental conditions. In our studies, LHRH immunoneutralization was chosen because of the specificity of this approach and its proven effectiveness in inducing gonadotropin deficiency in other species. Active immunization against LHRH results in testicular involution and decreased serum testosterone concentrations in the rat (Fraser et al, 1974b), rabbit (Arimura et al, 1973), ram (Schanbacher, 1982), bull (Robertson et al, 1979), and monkey (Chappel et al, 1980).

The dog was chosen as a model for induced gonadotropin deficiency since both hypothalamic-pituitary-gonadal interrelationships (Jones and Boyns, 1974; DePalatis et al, 1978; Falvo et al, 1979) and spermatogenesis (Foote et al, 1972) have been thoroughly studied in this species. Furthermore, the size of these animals, their availability, and

Reprint requests: Dr. B. D. Schanbacher, Roman L. Hruska U.S. Meat Animal Research Center, U.S. Department of Agriculture, Clay Center, Nebraska 68933.

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their docility allow for frequent blood sampling and pulsatile administration of gonadotropins under experimental conditions. The study herein reports on pituitary and testicular hormone secretion in adult male mongrel dogs following active immunization against LHRH. Changes in testis and prostate weights were used as additional indices of the effectiveness of LHRH immunization.

Materials and Methods

Animals

Ten sexually mature male mongrel dogs weighing 16 to 22 kg, and having normal palpable testes, were used in the present study. The dogs were housed in individual cages in a controlled environment (14L:10D photoperiod and 18–20 C) at the Milton S. Hershey Animal Care Facility and were assigned to one of two groups: LHRH-immunized dogs (A–E) and control dogs (F–J). Water and Purina Dog Chow were provided *ad libitum* during a 2-week acclimation period and throughout the 12-week experimental period.

Immunization Against LHRH

LHRH that had been conjugated to human serum albumin (hSA) by the carbodiimide reaction (Fraser et al, 1974a) was emulsified by sonication in equal volumes of sterile saline and Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). Dogs A–E were each administered subcutaneously 200 μg of the LHRH-hSA conjugate as described previously for ram lambs (Schanbacher, 1982). Booster injections of 200 μg LHRH-hSA in Freund's incomplete adjuvant were given to each dog at four and eight weeks after the initial immunization.

Antibody Titers to LHRH

Antibody titers to LHRH were determined in serum pools from each dog, before (week 0) and after (week 4, 6, 8, 10, and 12) LHRH immunization, by the following procedure. Serum pools from each of the immunized dogs were diluted 1:100 and 1:1000 with phosphate buffered saline containing 0.2% gelatin, whereas sera collected from control dogs at the end of the study were diluted 1:100 only. The diluted sera (400 μl in duplicate) were placed in 12 × 75 mm culture tubes with 20,000 cpm of ^{125}I-LHRH (100 μl; 1500 Ci/mol) and incubated over night at 4 C. The radioiodinated LHRH used for binding was prepared by reacting 2.5 μg LHRH with 0.5 mCi of ^{125}I (New England Nuclear, Boston, Massachusetts) and 4 μg of chloramine T. Mono-iodinated LHRH was separated from the other reaction products by QAE Sephadex chromatography (Nett and Adams, 1977). Free and bound ^{125}I-LHRH were separated by the addition of 100 μl of dextran-treated charcoal. Following centrifugation, the supernatants were transferred to 12 × 75 mm culture tubes and counted in a Packard gamma spectrometer (3365). Nonspecific binding, determined for the pre-immunization serum pool from each dog, was subtracted to estimate the percentage of ^{125}I-LHRH specifically bound (% B/Bo) in serum of LHRH-immunized dogs.

Pituitary and Testicular Responses

Peripheral blood samples were collected from dogs A–E at 20-minute intervals for six hours via indwelling external jugular catheters immediately before LHRH immunization, and again at the end of the study (week 12). Additional samples (n = 3) were collected via jugular catheters at 20-minute intervals during weeks 4, 6, 8, and 10 of the study. Serum concentrations of LH (DePalatis et al, 1978), FSH (Winter et al, 1982) and testosterone (Schanbacher and D'Occhio, 1982) were determined in duplicate within single assays by previously described double antibody radioimmunoassay procedures. Sensitivities for the respective assays were 0.5 ng LER-1685-1/ml for LH, 62.5 ng LER-1685-3A/ml for FSH, and 0.1 ng/ml for testosterone.

Twelve weeks after immunization, the five immunized dogs (A–E) and five control dogs (F–J) were weighed and sacrificed by euthanasia. The pituitary, testes and prostate of each animal were collected, weighed, and retained for histological examination.

Pituitary Immunocytochemistry

The pituitary glands of dogs A–E and G–I were fixed by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and embedded in Epon 812. Sections were processed for light microscopic immunocytochemistry according to the peroxidase-antiperoxidase procedure (Sternberger et al, 1970) as modified for use on plastic-embedded, osmium-fixed tissues (Erlandsen et al, 1979). Gonadotropes were identified using an antiserum generated against ovine LH. Immunoabsorption controls using a variety of purified hormones (Baker and Gross, 1978) demonstrated the high degree of specificity of this antiserum. At least nine sections taken from varying levels of the pars distalis were examined for each dog. The number of immunoreactive gonadotropes per section showing a distinct nuclear profile was counted and expressed as a function of the area of tissue in the section. Additionally, the cross-sectional area of these cells was determined as follows: two random fields per section were photographed and printed at a final magnification of 1680×. Area of individual cells was determined with a Zeiss Videoplan digitizing system, which provides automated, computer-interfaced analysis of the area of cells outlined with an electronic cursor. Differences between immunized and control groups were determined by Student's *t* test.

Results

Antibody Response to LHRH Immunization

The LHRH antibody titers were less than 3% in sera of all dogs before LHRH immunization. This was considered to represent nonspecific binding,

TABLE 1. LHRH Antibody Titers, Paired Testes Weights and Prostate Weights in Control and LHRH-immunized Mongrel Dogs

Treatment	Animal Weight (kg)	Antibody Titers (% B/Bo)*	Testes Weight (gm)	Testes Weight (gm/kgBW)	Prostate Weight (gm)	Prostate Weight (gm/kgBW)
Immunized Dogs						
Affected						
C	19.5	51.2	4.0	0.21	2.1	0.11
D	16.6	28.5	4.0	0.24	1.7	0.10
Unaffected						
A	19.7	14.2	14.4	0.75	13.4	0.70
B	22.7	8.1	18.4	0.81	15.1	0.67
E	19.9	9.8	16.8	0.84	15.3	0.77
Control Dogs						
F	15.5	ND	15.4	0.99	11.3	0.73
G	17.3	ND	10.6	0.61	15.8	0.91
H	16.0	ND	10.0	0.63	7.2	0.45
I	16.8	ND	11.7	0.70	5.1	0.30
J	16.0	ND	12.7	0.79	6.6	0.41
$\bar{X}$	16.3	—	12.1	0.74	9.2	0.56
± SEM	± 0.3		± 1.0	± 0.07	± 1.9	± 0.11

* Percentage of [^{125}I]-LHRH binding in serum diluted 1:10^3.
ND = nondetectable (<3% at 1:10^2 dilution).

and was therefore subtracted from the LHRH binding by sera of immunized dogs.

Titers of LHRH antibodies increased at different rates in each of the immunized dogs, with the highest titers observed in dog C, and the lowest titers observed in dog B (Table 1). Except for dog C, the immunized dogs had higher binding titers during weeks 6 and 10 (ie, 2 weeks after the booster injections) than during weeks 8 and 12 (ie, 4 weeks after the booster injections).

Endocrine Responses to LHRH Immunization

Mean serum concentrations of LH, FSH, and testosterone are presented for the five immunized dogs in Table 2. Before immunization, LH and testosterone concentrations fluctuated markedly during the six-hour bleed (Fig. 1, left panel), whereas FSH concentrations were relatively static (data not shown). Conspicuous LH peaks were observed in each of the dogs, and in most cases, these were followed by transient rises in serum testosterone. The greater number and amplitude of LH peaks observed in dogs B and C resulted in higher mean LH concentrations for these dogs, but mean serum testosterone concentrations were not elevated.

With the exception of an increase in mean serum testosterone in dog B during the last bleed (Table

TABLE 2. Serum Hormone Concentrations in Intact Mongrel Dogs Before and After Active Immunization Against Luteinizing Hormone Releasing Hormone (LHRH)

Dog	LH (ng/ml) Before	LH (ng/ml) After	FSH (ng/ml) Before	FSH (ng/ml) After	Testosterone (ng/ml) Before	Testosterone (ng/ml) After
A	4.4	2.7	112	120	2.9	2.6
B	9.1	11.4	119	94	1.8	7.8*
C	11.7	ND*	148	92*	1.4	0.3*
D	4.4	ND*	107	90	1.9	0.1*
E	5.9	5.1	120	124	2.8	2.4

Values are mean concentrations for a 6-hour intensive bleed the day before and 12 weeks after LHRH immunization.
ND = nondetectable (<0.5 ng/ml).
* $P < 0.01$. Significantly different from pre-immunization concentration (paired *t* test).

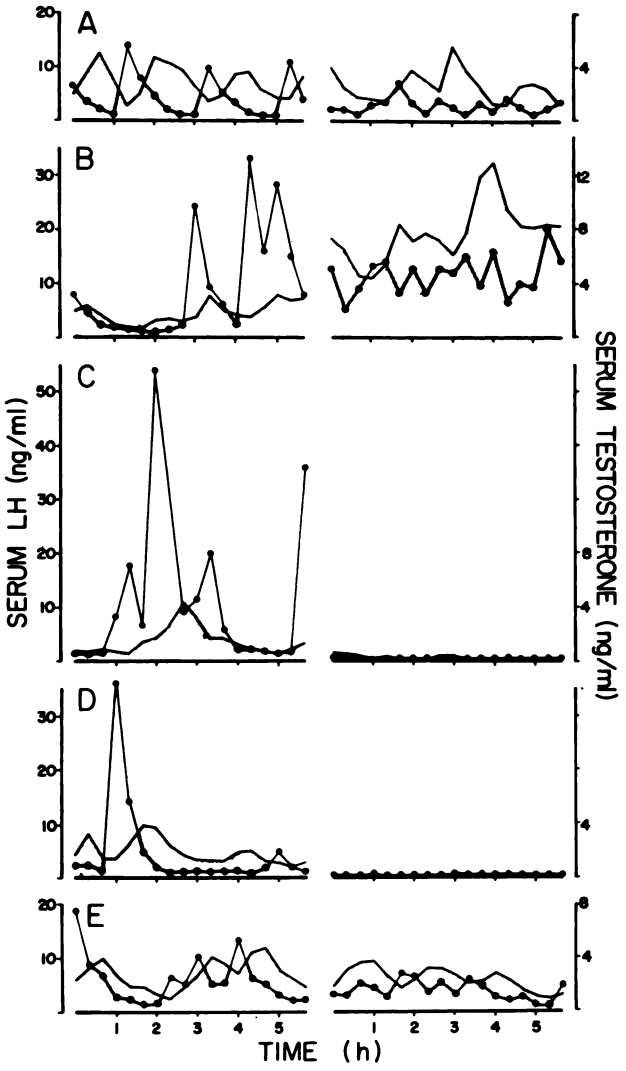

Fig. 1. Serum LH (●) and testosterone (—, shaded area) profiles in five mongrel dogs (A–E) one day before (left panel), and 12 weeks after (right panel) LHRH immunization.

2 and Fig. 1), marked changes were not observed in the endocrine parameters of dogs A, B, and E during the course of this study. Dogs C and D, on the other hand, had low serum LH concentrations (<0.8 ng/ml) at 10 and 12 weeks, and low serum testosterone concentrations (<0.6 ng/ml) as early as week 8. Figure 1 contrasts the secretory profiles of dogs C and D before and after immunocastration was imposed. Serum FSH was significantly reduced in dog C at the end of the study; however, the reduction was small in comparison to that observed for serum LH.

Pituitary Gonadotropes

No changes were noted in the size, number, distribution, or staining intensity of the immunoreactive gonadotropes of dogs A, B, and E as compared to the control dogs. However, gonadotropes from dogs C and D were considerably smaller, fewer in number, and less intensely stained than control cells (Fig. 2, Table 3).

Testes and Prostate Responses to LHRH Immunization

Weights of both LHRH-immunized and control dogs at the time of sacrifice are presented in Table 1, along with testis and prostate weights. Because of weight differences between individual dogs, testis and prostate weights are also expressed as a function of body weight. Whether expressed in absolute weight or as a function of body weight, the testes and prostates of dogs C and D were conspicuously small, ie, approximately one-fourth (for testes) and one-seventh (for prostate) the weights of control tissues. The heavier weights of the testis and prostates from immunized dogs, A, B, and E, and control dogs, F–J, suggest that antibody titers in the immunized group were either inadequate or not of sufficient affinity to induce testicular involution.

Discussion

The present study describes the pituitary and testicular endocrine responses of intact male mongrel dogs to active immunization against LHRH. The results demonstrate that antibodies to LHRH can be generated in this species by injection of microgram quantities of LHRH conjugated to human serum albumin. When produced in sufficient amounts, the LHRH antibodies can effectively lower LH levels, presumably through immunoneutralization of endogenous LHRH. In turn, the pituitary gonadotropes regress, serum concentrations of LH and testosterone are decreased, testis size is diminished, and weight of the prostate gland is reduced.

The hormonal and tissue responses observed in dogs C and D closely resembled the findings in patients with incomplete isolated gonadotropin deficiency. These subjects have undetectable levels of LH, low testosterone levels, and reduced testis size (Santen and Paulsen, 1973a,b; Santen and Kulin, 1981). On the other hand, FSH is affected to a lesser extent in isolated gonadotropin defi-

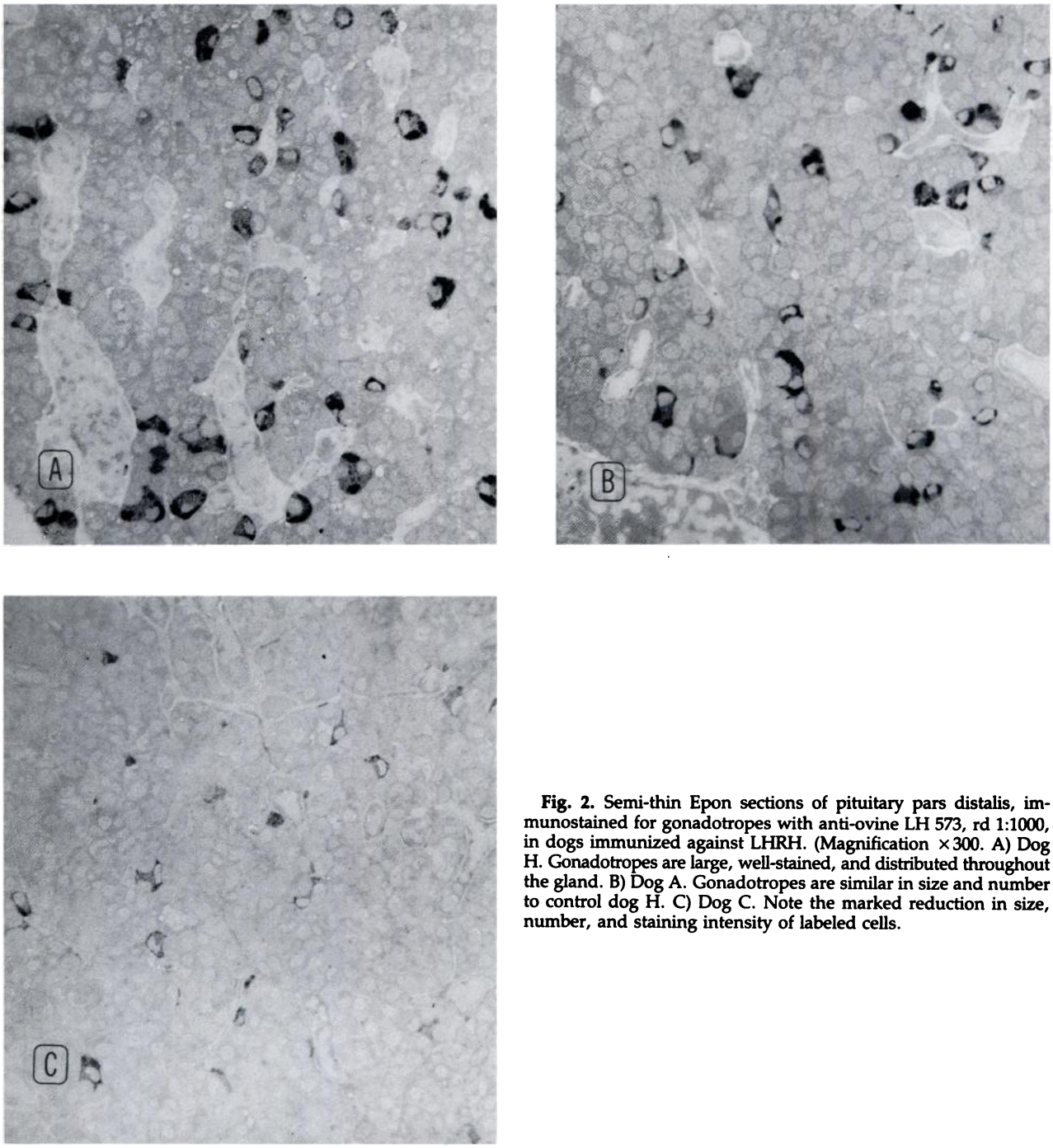

Fig. 2. Semi-thin Epon sections of pituitary pars distalis, immunostained for gonadotropes with anti-ovine LH 573, rd 1:1000, in dogs immunized against LHRH. (Magnification ×300. A) Dog H. Gonadotropes are large, well-stained, and distributed throughout the gland. B) Dog A. Gonadotropes are similar in size and number to control dog H. C) Dog C. Note the marked reduction in size, number, and staining intensity of labeled cells.

ciency, as in the two dogs effectively immunized against LHRH (Santen et al, 1971; Santen and Paulsen, 1973b; Williams et al, 1975). Since isolated gonadotropin deficiency in man appears to be secondary to LHRH deficiency, it was anticipated that LHRH immunoneutralization in dogs would produce similar effects. Although the responses in individually immunized dogs were variable, the affected dogs would appear to serve as useful experimental models to evaluate the responses to pulsatile and constant modes of gonadotropin replacement therapy.

TABLE 3. Size and Distribution of Gonadotropes in the Pituitaries of Control and LHRH-immunized Mongrel Dogs

Treatment	Size of LH Immunoreactive Gonadotropes (μ^2)	Number of LH Immunoreactive Gonadotropes (#/$\mu^2 \times 10^{-5}$)
Immunized Dogs		
Affected		
C	101 ± 26	35 ± 7
D	100 ± 24	48 ± 6
Unaffected		
ABE	185 ± 18	79 ± 13
Control Dogs		
G-I	203 ± 22	78 ± 11

Values are means ± SE.

Findings similar to those described above have been reported for LHRH-immunized males of other species. Fraser et al (1974b) reported that active immunization to LHRH results in atrophy of the testes and secondary reproductive organs and concomitant aspermatogenesis in male rats. Decreased serum LH and gonadal atrophy have also been reported in a proportion of rabbits (Arimura et al, 1973) and male rhesus monkeys (Chappel et al, 1980) immunized against LHRH. Immunization of ram lambs against the same antigen as was used in the present study with dogs (LHRH-hSA), resulted in a "castration response" in all lambs (Schanbacher, 1982), suggesting that lambs are more susceptible to the immunocastration procedure. Although it is possible that lambs are more susceptible to the immunoneuralizing effects of LHRH antibodies, or that species differences exist for the neuroendocrine requirements of pituitary gonadotropin secretion, the more plansible explanation is that dogs simply did not respond adequately to the immunological challenge. Alternatively, differences between the two studies may be related to differences in the neuroendocrine inputs necessary for normal testicular function between pre- and postpubertal animals.

The production of high titer LHRH antisera and the induction of a "hypophysectomy-like response" in immunized dogs C and D provide incentive for further development of this immunological approach to producing gonadotropin deficiency. Even though antibody titers were measured in dogs A, B, and E following LHRH immunization, serum hormone concentrations and testes weights were normal. An inverse relationship between LHRH antibody titers and serum concentrations of reproductive hormones has been reported in immunized male rats (Fraser et al, 1974b), rhesus macaques (Chappel et al, 1980), and bulls (Robertson et al, 1981). Based on previous findings (Fraser (1982), and those reported in the present study, it is suggested that LHRH antibody titers in excess of 25% B/Bo at 1:1000 dilution are required to provide effective immunoneutralization of LHRH in the dog. To improve on the success rate of immunohypophysectomy, and at the same time conserve on the amounts of purified immunogen required for successful immunization, an appropriate combination of potent immunogen with potent and safe immunologic adjuvants is required. While the potent and commonly used Freund's complete adjuvant has been utilized in all of the LHRH immunization experiments to date, the question of adjuvant safety necessitates the testing and implementation of new and acceptable adjuvants.

LHRH immunoneutralization could also be employed as an effective sterilant for the control of populations of dogs and other pets. However, modifications of the present technique are required before testing commercially. The minimal number of booster injections required for sterilization should be known, as well as the reversibility of this procedure. The observed decrease in the antibody titers between 2 and 4 weeks after booster injections in the present study, and the similar decrease reported in rhesus macaques (Chappel et al, 1980) and bulls (Robertson et al, 1981) following booster injections, suggests that repeated exposure to immunogen will be required.

Results of this study indicate 1) that normal testicular function in sexually mature mongrel dogs requires a continuous supply of the neurohormone, LHRH (Jones et al, 1976), and 2) that neutralization of endogenous LHRH by active immunization may provide a suitable alternative to surgical castration in the dog.

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